Contents

Introduction

The E.Z.N.A.® Mollusc RNA Kit is designed for efficient recovery of total RNA greater than 200 nt from molluscs, arthropods, roundworms, flatworms, and other invertebrate tissue samples rich in mucopolysaccharides. The procedure relies on the well established properties of the cationic detergent, cetyltrimethyl ammonium bromide (CTAB), in conjunction with the selective RNA binding of Omega Bio-tek's HiBind® matrix. Samples are homogenized and lysed in a high salt buffer containing CTAB and extracted with chloroform to remove mucopolysaccharides and denature proteins. Following a rapid alcohol precipitation step, binding conditions are adjusted and RNA further purified using HiBind RNA spin columns. In this way salts, proteins and other contaminants are removed to yield high quality total RNA suitable for downstream applications such as reverse transcription, poly (A)+ mRNA selection, and hybridization techniques.

New in this edition

- On-column DNase I digestion protocol included. (Page 5)
- New capped V-spin column available in this kit (kit # R6875)

Storage and Stability

All components of the Mollusc RNA Kit are stable for up to 24 months from the date of purchase when stored at 22°C-25°C. During shipment or storage in cool ambient conditions, salts may precipitate in certain buffers. Simply warm to 37°C and mix to dissolve. Contents of the kit should not be refrigerated at any time.

Kit Contents

Product	R6675-00 R6875-00	R6675-01 R6875-01	R6675-02 R6875-02
Preps	5	50	200
HiBind® RNA Columns	5	50	200
2 ml Collecting tubes	10	100	400
Buffer MRL	2 ml	20 ml	80 ml
Buffer RB	5 ml	30 ml	100 ml
RNA Wash Buffer I	5 ml	45 ml	175 ml
RNA Wash Buffer II Concentrate	5 ml	12 ml	45 ml
DEPC water	1 ml	20 ml	50 ml
User Manual	1	1	1

Before Starting

- RNA Wash Buffer II is supplied as a concentrate and must be diluted with absolute ethanol before first use. Add 20 ml *(R6675-00, R6876-00)*; 48 ml *(R6675-01, R6875-01)*; or 180 ml *(R6675-02, R6875-02 prep)* ethanol to each bottle of RNA Wash Buffer II Concentrate as indicated on the label. Once diluted with ethanol, RNA Wash Buffer must be stored at room temperature.

- Buffer RB contains a chaotropic agent. Handle with care, always wearing disposable latex gloves and appropriate protective eye-ware.

Working with RNA

Please take a few minutes to read this booklet thoroughly to become familiar with the protocol. Prepare all materials required before starting to minimize RNA degradation.

- Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Change gloves frequently. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.

- During the procedure work carefully but quickly.

- Under cool ambient conditions, crystals may form in buffers. This is normal and the bottle may be warmed to redissolve the salt.

- 2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing endogenous RNases and must be added to an aliquot of Buffer MRL and Buffer RB before use. Add 20 µl of 2-mercaptoethanol per 1 ml of Buffer RB or

MRL. This mixture can be stored for 1 week at room temperature.

E.Z.N.A.™ Mollusc/Arthropod RNA Protocol

Materials to be provided by user

- Microcentrifuge capable of 10,000 x g
- Nuclease-free microfuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol)
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (100 µl per sample) of DEPC-treated water at 65°C.

NOTE: Use extreme caution when handling liquid nitrogen.

Use only fresh tissue to ensure RNA integrity. Samples preserved in formalin usually yield degraded RNA but may still yield adequate results for RT-PCR of target regions <500 nt.

Arthropods

1. Pulverize no more than 50 mg of tissue in liquid nitrogen with mortar and pestle and place the powder in a clean 1.5 ml microcentrifuge tube. If ceramic mortar and pestle are not available, homogenize the sample in the microfuge tube using a disposable microtube pestle (OBI Cat# SSI-1014-39 & SSI-1015-39).

Proceed to step 2 below.

Molluscs (and other soft tissued invertebrates)

1. Grind no more than 30 mg tissue in liquid nitrogen with mortar and pestle and place the powder in a clean 1.5 ml microcentrifuge tube. If ceramic mortar and pestle are not available, homogenize the sample in the microfuge tube using a disposable microtube pestle (OBI Cat# SSI-1014-39 & SSI-1015-39).

Amount of starting material depends on sample and can be increased if acceptable results are obtained with the suggested 30 mg tissue. For easy to

process specimens, the procedure may be scaled up and the volumes of all buffers used increased in proportion. In any event, use no more than 50 mg tissue per HiBind® spin-column as RNA binding capacity (100 µg) may be exceeded. Meanwhile, difficult tissues may require starting with less than 30 mg tissue and doubling all volumes to ensure adequate lysis.

Proceed with step 2 below.

2. Add 350 µl Buffer MRL./2-mercaptoethanol and vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.
   Note: Add 20 µl 2-mercaptoethanol per 1 ml of Buffer MRL before use. This mixture can be made and stored at room temperature for 1 week.

3. To the lysate add 350 µl chloroform:isoamyl alcohol (24:1) and vortex to mix. Centrifuge 10,000 x g for 2 min at room temperature. Carefully transfer the **upper** aqueous phase to a clean 1.5 ml microfuge tube. Avoid the milky interface containing contaminants and inhibitors.
   Note: This step will remove much of the polysaccharides and proteins from solution and improve spin-column performance downstream.

4. Add one volume of isopropanol and mix to precipitate RNA. Immediately centrifuge 10,000 x g for 2 min at room temperature. Carefully discard as much supernatant as possible without disturbing RNA pellet. Invert the microfuge tube on a paper towel for 1 min to allow residual liquid to drain; it is not necessary to dry the pellet.

5. Add 100 µl of sterile DEPC-treated water or RB buffer (supplied) pre-heated to 65°C and vortex to resuspend the pellet. A brief incubation at 65°C may be necessary to effectively dissolve the RNA.
   Note: RB buffer recommended at this step to avoid RNA degradation.

6. Adjust binding conditions by adding 350 µl Buffer RB/2-mercaptoethanol followed by 250 µl absolute ethanol and vortex to mix. Apply entire mixture, including any precipitation that may have formed, to an HiBind® RNA column assembled in a 2 ml collecting tube (supplied).

7. Centrifuge 10,000 x g for 15 sec at room temperature. Discard flow-through liquid and reuse collecting tube in next step.
   Note: Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RB before use. This mixture can be made and stored at room temperature for 1 week.

8. Add 500 µl RNA Wash Buffer I and centrifuge at 10,000 x g for 15 sec. Discard both flow-through liquid and collecting tube.

9. Place column in a clean 2 ml collection tube (supplied), and add 500 µl Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 15 sec at room temperature and discard flow-through. Reuse the collection tube in step 9.
   Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

10. Wash column with a second 500 µl of Wash Buffer II as in step 8. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind™ matrix.

11. Elution of RNA. Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. A second elution into the same tube may be necessary if the expected yield of RNA >50 µg. To maintain a high RNA concentration, use the first eluate for the second elution.
    Note: RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution.

Short Protocol

The following short protocol is designed for isolating RNA from mollusc and most soft tissued invertebrate.

1. Grind no more than 30 mg tissue in liquid nitrogen with mortar and pestle and place the powder in a clean 1.5 ml microcentrifuge tube. If ceramic mortar and pestle are not available, homogenize the sample in the microfuge tube using a disposable microtube pestle (OBI Cat# SSI-1014-39 & SSI-1015-39).

2. Add 350 µl Buffer RB./2-mercaptoethanol and vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

3. Centrifuge 10,000 x g for 5 min at room temperature. Carefully transfer the **supernatant** to a clean 1.5 ml microfuge tube. Avoid the milky interface containing contaminants and inhibitors.

4. Adjust binding by adding 350 µl 70% ethanol and vortex to mix. Apply entire mixture, including any precipitation that may have formed, to an HiBind® RNA column assembled in a 2 ml

collecting tube (supplied).

5. Add 500 µl RNA Wash Buffer I and centrifuge at 10,000 x g for 15 sec. Discard both flow-through liquid and collecting tube.

6. Place column in a clean 2ml collection tube (supplied), and add 500 µl Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 15 sec at room temperature and discard flow-through. Reuse the collection tube in step 7.

7. Wash column with a second 500 µl of Wash Buffer II as in step 8. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind™ matrix.

8. Elution of RNA. Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. A second elution into the same tube may be necessary if the expected yield of RNA >50 µg. To maintain a high RNA concentration, use the first eluate for the second elution.

DNase digestion Protocol (Optional)
Since HiBind® RNA resin and spin-column technology actually removes most of DNA without the DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion:(see DNase I cat.# E1091 for detail information)

1. Follow the standard protocol until the samples completely pass through the HiBind RNA column (step1-7). Prepare the following:

a. For each HiBind® RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz unites/µl)	1.5 µl
Total volume	75 µl

Note:
1. **DNase I is very sensitive for physical denaturaion, so do not vortex this DNase I mixture. Mix gently by inverting the tube. Prepare the fresh DNase I digestion mixture before RNA isolation.**
2. **OBI DNase I digestion buffer is supplied with OBI RNase-free Dnase set.**
3. **Standard Dnase buffers are not compatible with on-membrane Dnase digestion.**

b. Pipet 75 µl of the DNase I digestion reaction mix directly onto the surface of HiBind® RNA resin in each column. Make sure to pipet the Dnase I digestion mixture directly onto the membrane. Dnase I digestion will not be complete if some of the mix stick to the wall or the O-ring of the HiBind® RNA column.

c. Incubate at room temperature(25-30°C) for 15 minutes

2. **Place column in a clean 2ml collection tube**, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge and discard flow-through. Reuse the collection tube in step 7.

3. **Place column in the same 2ml collection tube**, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through. Reuse the collection tube in step 7.
Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

4. Wash column with a second 500 µl of Wash Buffer II as in step 5. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind® matrix.

5. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. A second elution may be necessary if the expected yield of RNA >50 µg.
Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

Quantitation and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance at 260 nm and 280 nm in a spectrophotometer. 1 O.D. unit measured at 260 nm corresponds to 40 µg of RNA per ml. The ratio of A_{260}/A_{280} of pure nucleic

acids is 2.0, while for pure protein it is approximately 0.6. A ratio of 1.8-2.0 corresponds to 90%-100% pure nucleic acid. Store RNA samples at -70°C in water. Under such conditions RNA prepared with the E.Z.N.A. system is stable for more than a year.

RNA Quality

It is highly recommended that RNA quality be determined prior to all analyses. The quality of RNA can be assessed by denaturing agarose gel electrophoresis and ethidium bromide staining. Several sharp bands should appear on the gel. These are the 28S and 18S ribosomal RNA bands as well as certain populations of mRNA and possibly viral RNA bands. If these bands smear towards lower molecular weight RNAs, then the RNA has undergone major degradation during preparation, handling, or storage. RNA molecules less than 200 bases in length do not efficiently bind the HiBind matrix, thus the method enriches high quality mRNA.

Troubleshooting Guide

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	• Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution. • Incubate column for 2 min with water prior to centrifugation.
	Column is overloaded	• Reduce quantity of starting material.
Clogged column	Incomplete disruption or lysis of tissue.	• Completely disrupt sample in liquid nitrogen. • Increase centrifugation time. • Reduce amount of starting material
Precipitated RNA will not dissolve.	High nucleic acid and polysaccharide content.	• Reduce amount of starting material. Generally it is best to start with 50-100 mg at first. • To avoid RNA degradation, do not increase incubation time for resuspension.
Degraded RNA	Source	• Freeze starting material quickly in liquid nitrogen and store at -70°C without thawing. • Follow protocol closely, and work quickly. • Make sure that 2-mercaptoethanol is added to Buffer MRL. • Try short protocol
	RNase contamination	• Ensure not to introduce RNase during the procedure. • Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	• Ensure Wash Buffer II has been diluted with 100% ethanol as indicated on bottle. • Diluted Wash Buffer II must be stored at room temperature. • Repeat wash with Wash Buffer II.
DNA contamination	Co-purification of DNA	• Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	• DEPC-treated water is acidic and can dramatically lower Abs260 values. Use TE buffer (pH 8) to dilute RNA prior to spec analysis.

Order Information

Product Number	Product Name	Description
E.Z.N.A.™ Total RNA Miniprep Kits		
R6634-01/02 R6834-01/02	E.Z.N.A.™ Total RNA Kit	Total RNA isolation from animal cells or tissues.
R6627-01/02 R6827-01/02	E.Z.N.A.™ Plant RNA Kit	Total RNA Isolation from plant samples
R6640-01/02 R6840-01/02	E.Z.N.A.™ Fungal RNA Kit	Total RNA Isolation from fungal samples
R6670-01/02 R6870-01/02	E.Z.N.A.™ Yeast RNA Kit	Total RNA Isolation from yeast samples
R6850-01/02 R6950-01/02	E.Z.N.A.™ Bacterial RNA Kit	Total RNA Isolation from yeast samples
R6675-01/02 R6875-01/02	E.Z.N.A.™ Mollusc RNA Kit	Total RNA Isolation from mollusc, invertebrates samples.

E.Z.N.A.™ Total RNA Midi/maxi Kits		
R6664-01/02	E.Z.N.A.™ Total RNA Midi Kit	Total RNA isolation from animal cells or tissues
R6693-01/02	E.Z.N.A.™ Total RNA Maxi Kit	Total RNA isolation from animal cells or tissues
R6615-01/02	E.Z.N.A.™ Blood RNA Midi Kit	Total RNA isolation from blood samples
R6616-01/02	E.Z.N.A.™ Blood RNA Maxi Kit	Total RNA isolation from blood samples
R6628-01/02	E.Z.N.A.™ Plant RNA Midi Kit	Total RNA isolation from plant samples
Other RNA isolation kit, Reagent and supplies		
R6511-01/02	mRNA Enrichment kit	mRNA isolation
R6830-01/02	RNA-Solv ™ reagent	Single reagent for total RNA isolation
R6248-01/02 R6249-01/02	E.Z.N.A.™ RNA Probe purification kit	RNA Probe purification
R6376-01/02	E.Z.N.A.™ Poly-Gel RNA Isolation Kit	Isolate RNA from poly-acrylamide gel
R6500-01/02	E.Z.N.A.™ Oligo (dT) Cellulose	High capacity oligo(dT) cellulose